

Frequent Down-Regulation of E-cadherin by Genetic and Epigenetic Changes in the Malignant Progression of Hepatocellular Carcinomas¹

Takuya Matsumura, Reiko Makino,² and Keiji Mitamura

Second Department of Internal Medicine, Showa University School of Medicine, Tokyo, 142-8666, Japan

ABSTRACT

E-cadherin mediates cell-cell adhesion by associating with catenins. Loss of E-cadherin function by genetic or epigenetic alteration of the E-cadherin gene (*CDH1*) leads to tumorigenesis. To study the involvement of E-cadherin dysfunction in liver tumorigenesis, we examined the allelic loss and methylation of 5'-CpG sites of *CDH1* in hepatocellular carcinomas (HCCs). Loss of heterozygosity (LOH) of *CDH1* and adjacent 16q22-23 loci was observed in 13 of 30 (43%) HCCs. Methylation of the 5'-CpG of *CDH1* was analyzed by Southern blot hybridization, and hypermethylation was observed in 8 of the 24 (33%) HCCs examined. The amount of E-cadherin mRNA was analyzed by RNase protection assay, and a decrease in E-cadherin mRNA was observed in 10 of the 23 cases examined. A reduction in E-cadherin was found in 10 of 21 HCCs using immunoblot analysis. The amount of E-cadherin was comparable to that of E-cadherin mRNA. Down-regulation of E-cadherin was common in cases with LOH but rare in cases with methylated promoter. These results suggest that hypermethylation of the *CDH1* promoter is present in a small cell population in the tumor, thus the methylation status is liable to vary according to individual cell condition. Hypermethylation was observed in early stage HCCs, whereas LOH was found frequently in more malignant tumors. Down-regulation of E-cadherin is closely related to the progression of HCCs and is stably induced by LOH of *CDH1*.

INTRODUCTION

HCC³ is common in Asia and has recently been found to be increasingly common in the United States (1). In many cases, HCC develops from chronic liver disease by infection with HBV or HCV or by exposure to drugs such as aflatoxin B1. The molecular mechanisms of HCC induction are not yet clear. An AGG (Arg) to AGT (Ser) mutation of p53 codon 249 is often found in aflatoxin-mediated HCCs (2-4); however, this is rare in Japanese cases (5-7). Mutation of the *p53* gene is found in 30-40% of HCCs in Japan (4-7), which is not a high frequency compared to the rates for other types of cancer. Mutation of the β -catenin gene is found in 30-40% of HCCs, which indicates that changes in the Wnt signaling pathway are important in hepatocellular carcinogenesis (8, 9). E-cadherin mediates cell-cell adhesion by association with intracellular molecules of α -, β -, and γ -catenins (10). Reduction of E-cadherin may induce cell mobility and promote tumor cell invasion (11). Recently, loss of E-cadherin function has been observed in the malignant progression of various tumors originating from epithelial cells. *CDH1* is localized on chromosome 16q22. Germ-line mutation of *CDH1* is found in familial diffuse-type gastric cancers (12). Somatic mutations of *CDH1* are found in poorly differentiated breast and gastric cancers (13-15) but rarely found in other types of tumors. On the other hand, down-regulation of *CDH1* with hypermethylation of the CpG islands of the region promoter is observed in a wide variety of tumors originating from epithelial cells (16, 17). Therefore, reduction of E-cadherin expression induces and promotes cellular tumorigenesis. Reduced expression of *CDH1* by hypermethylation of the 5'-CpG island of E-cadherin has been observed in HCCs (18). Furthermore, the allelic loss of E-cadherin has also been observed frequently in HBV-positive HCCs in China (19). However, there has been no comprehensive study of genetic and epigenetic changes and down-regulation of E-cadherin in HCCs.

In this study, we examine genetic and epigenetic alterations of *CDH1* and the resulting changes in *CDH1* expression to determine the involvement of E-cadherin dysfunction in liver tumorigenesis. We have found that the down-regulation of E-cadherin by allelic loss is closely related to the progression of HCCs.

MATERIALS AND METHODS

Tissue Specimens. Thirty pairs of surgically resected primary HCCs and adjacent nontumorous liver tissues and two

Received 8/14/00; revised 11/16/00; accepted 12/12/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported in part by funds for the High-Technology Research Center Project from the Ministry of Education, Science, Sports and Culture of Japan.

² To whom requests for reprints should be addressed, at Second Department of Internal Medicine, Showa University School of Medicine, Hatanodai 1-5-8, Shinagawa-ku, Tokyo, 142-8666, Japan. Phone: 81-3-3784-8225; Fax: 81-3-3784-7553; E-mail: rmakino@med.showa-u.ac.jp.

³ The abbreviations used are: HCC, hepatocellular carcinoma; *CDH1*, E-cadherin gene; LOH, loss of heterozygosity; HBV, hepatitis B virus; HCV, hepatitis C virus; SSCP, single-strand conformational polymorphism; SNP, single nucleotide polymorphism.

normal liver tissues were used. The tumor stages of HCC were classified according to the tumor-node-metastasis (TNM) criteria (20). Nontumorous livers of these patients were affected by chronic hepatitis in 10 cases, liver cirrhosis in 16 cases, and other disease in 4 cases. These tissues were stored at -80°C until use. This study was approved by the Ethical Review Committee of the Showa University School of Medicine (Tokyo, Japan).

LOH Analysis of *CDH1* and 16q22–23 Loci. Genomic DNA was extracted by the proteinase K-phenol/chloroform method. DNA was extracted from HCCs and adjacent nontumorous liver tissues. The LOH of *CDH1* was determined by fluorescence-based PCR-SSCP analysis (7) using SNP markers at 2076 bp downstream (codon 692) (21) and at 285 (22) and 472 bp upstream from the translation initiation site. The primer sequences were as follows: (a) CTCCAGCCCAAGAATC-TATC and CTGGACTTACTTAGCAAAGC (+2076); (b) GGAATCAGAACCGTGCAGGT and AGCGCCGAGAGGC-TGCGGCT (–285); and (c) AGGAGTTCGAGGCTGCAGTG and CCCACCCGGCCTCGCATAGA (–472). The 5'-ends of the primers were labeled with Cy5 dye. PCR for +2076 was at 94°C for 1 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. PCR for –285 and –472 were at 95°C for 1 min, followed by 30 cycles at 95°C for 45 s, 65°C for 45 s, and 72°C for 1 min. PCR was performed with ExTaq DNA polymerase (Takara, Shiga, Japan). Five percent DMSO was added to the reaction mixture for amplification of the –285 and –472 regions. Amplified DNA fragments were electrophoresed and analyzed by an ALFexpress DNA sequencer and an ALFwin Fragment analyzer program (Pharmacia, Uppsala, Sweden), as described previously (7). Any HCC that showed a lesser or absent peak compared with the peak pattern of nontumorous tissue from the same patient was taken to reflect LOH.

For LOH analysis of 16q22–23 in the vicinity of *CDH1*, five microsatellite markers were prepared: (a) D16S3031; (b) D16S3021; (c) D16S496; (d) D16S3025; and (e) D16S2624 (23, 24). Detailed information including primer sequences is available on the web site of the Genome Database.⁴ The 5'-end of one of the paired primers was labeled with Cy5 dye, and PCR was performed as described previously (23, 24). Amplified Cy5-labeled DNA fragments were electrophoresed with 8% Long Ranger (FMC BioProducts, Rockland, ME) in $1\times$ Tris-borate EDTA containing 7 M urea by an ALFexpress automated DNA sequencer and analyzed by an ALFwin Fragment analyzer program. LOH in HCC was estimated by an absent or reduced peak compared to nontumorous liver from the same patient in a heterozygous case.

Methylation Analysis of the CpG Island of *CDH1* Promoter. Methylation of the 5'-CpG island of E-cadherin was analyzed using Southern blot analysis. Two μg of DNA were digested with *Bam*HI/*Xba*I, *Bam*HI/*Xba*I/*Msp*I, or *Bam*HI/*Xba*I/*Hpa*II. *Msp*I and *Hpa*II recognize the DNA sequence CCGG, but *Hpa*II does not cut the methylated cytosine. DNA fragments were separated by electrophoresis and then transferred to a nylon membrane (Hybond NX; Amersham). A plasmid contain-

ing *CDH1* promoter was kindly provided by Dr. J. A. Schalken (25). Hybridization was performed in $5\times$ SSC and 50% formamide at 42°C , using a ^{32}P -labeled, 378-bp *Pst*I fragment of the promoter region of *CDH1* as a probe.

Detection of E-cadherin mRNA by RNase Protection Analysis. Total RNA was extracted from frozen tissues using the AGPC method (26). Ten μg of total RNA were subjected to RNase protection assay using a HybSpeed RPA kit (Ambion, Austin, TX). The cDNA fragment from exons 9 to 10 (1295–1474 of *CDH1* cDNA; GenBank accession number NM004360) prepared by reverse transcription-PCR was subcloned into the pBluescript SK(+) plasmid. A RNA probe for detecting E-cadherin mRNA was prepared with [α - ^{32}P]UTP using T3 RNA polymerase. The probe protected the 180-nucleotide RNA fragment. The amount of E-cadherin mRNA was determined using a BAS image analyzer (Fuji Film, Tokyo, Japan). To assure the quality of mRNA, we also analyzed α -tubulin mRNA using a RNase protection assay.

Detection of E-cadherin Protein by Immunoblot Analysis. The cell extract was prepared as follows. Frozen tissues were homogenized with 5 volumes of lysis buffer [50 mM Tris-HCl (pH 7.5), 1% IGEPAL CA-630, (Sigma, St. Louis, MO) 0.1% sodium deoxycholate, 150 mM sodium chloride, and 1 mM phenylmethylsulfonyl fluoride] with a Teflon homogenizer. The cell debris was then precipitated by centrifugation ($2000\times g$ for 5 min). The protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL). Cell extracts containing 10 μg of protein were used to detect E-cadherin by immunoblot analysis. The monoclonal antibodies for human E-cadherin and α -tubulin were obtained from Takara and Santa Cruz Biotechnology (Santa Cruz, CA), respectively.

RESULTS

LOH of *CDH1* and 16q22–23 Loci in HCCs. The heterozygous deletion of *CDH1* and adjacent 16q22–23 loci was analyzed in 30 HCCs. Results are summarized in Fig. 1. We discovered a new SNP at 472 bp upstream from the translation initiation site. The major allele of this site is G, but a variant has A inserted here. We used this new SNP as a marker for the LOH analysis. Other markers used to analyze *CDH1* LOH were a C>T variant 2076 bp downstream of the initiation codon, which has been reported to be extremely heterozygous (21), and a C>A variant 285 bp upstream of the translation initiation site, which is identical to the variant 160 bp upstream of the transcription start site referred to by Li *et al.* (22). The SSCP pattern of the +2076 site is shown in Fig. 2A. Allelic loss of *CDH1* was found in 8 of 23 (35%) informative cases. We next analyzed the LOH of 16q22–23 in the vicinity of *CDH1* using five microsatellite markers (Fig. 2B). All of the nontumorous tissues of the liver adjacent to HCCs showed a heterozygous pattern with some of the microsatellite markers. The LOH of this locus was observed in 13 HCCs. By combining these data, we found allelic loss of *CDH1* in 13 of the 30 HCCs (43%).

The characteristics of HCCs and alterations of the *CDH1* gene are summarized in Table 1. The LOH of *CDH1* was observed at a higher frequency in more malignant HCCs. All of the stage III tumors carried allelic deletion of *CDH1*. The LOH of *CDH1* was found frequently in the moderately differentiated

⁴ www.ncbi.nlm.nih.gov/genome/sts.

Case	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30			
Tumor stage	I	II	I	II	II	II	III	III	II	I	II	II	II	II	II	IV	II	I	I	I	II	II	I	II	I	II	II	I	NI	II			
Chr. 16q	Pathology	M	M	M	P	M	M	M	M	M	M	M	M	W	W	M	P	P	W	M	M	W	W	W	M	W	M	M	W	M	P		
	Etiology	C	N	N	C	C	N	B	C	C	C	C	B	C	C	C	B	N	B	C	C	C	C	C	N	C	C	C	B	C	C		
cen																																	
	D16S3031					●					●		●	●					○	○	○				○			○	○	○	○		
	D16S3021	●	●	●	●	●	●	●	●	●	●	●			○	○			○	○	○	○	○	○	○	○	○	○	○	○	○	○	
	CDH1	●	●	●	●	●	●	●								○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	
	D16S496	●	●	●						●		●	●	●		○						○	○	○	○	○	○	○	○	○	○	○	
	D16S3025	●	●		●		●			●	●				○	○		○			○					○	○		○	○	○	○	
	D16S2624	●	●	●	●	●		●	●	●	●		●				○	○				○	○	○	○	○	○	○	○	○	○	○	○
tel																																	
Methylation of 5'-CpG	NT	-	-	-	-	-	-	-	-	-	-	-	-	-	ND	ND	-	-	-	-	ND	-	-	+	ND	-	ND	-	ND	-	-		
	HCC	+	-	-	-	-	-	-	-	+	+	-	-	-	+	ND	ND	+	+	+	-	ND	-	-	+	ND	-	ND	-	ND	-	-	
E-cad mRNA	NT	++	++	++	+++	++	++	ND	++	+++	++	++	++	++	++	ND	ND	ND	+++	++	ND	ND	++	++	+	+++	ND	++	+++	ND	++		
	HCC	+	++	+	+	+	+	ND	++	++	+	+	++	++	+	ND	++	ND	+++	++	ND	ND	++	++	+++	+++	ND	+	+++	ND	++		
E-cad protein	NT	++	++	+	ND	++	++	ND	++	++	++	++	+++	+++	+	ND	ND	++	ND	ND	ND	++	++	+	ND	ND	++	++	ND	+	+		
	HCC	+	+	+	ND	+	+	ND	++	+	+	+	++	+	++	++	ND	ND	+++	++	ND	ND	++	++	++	ND	ND	+	++	ND	++		
SNP	-285	1	1	1	1/2	1	1	1/2	1	1	1	1	1	1	1	1	1	1/2	1	1	1	1	1	1/2	1/2	1	1	1/2	1	1/2			

Fig. 1 Genetic analysis and expression of *CDH1* in HCCs. Markers are shown from the centromere (*cen*) to the telomere (*tel*) of the 16q22–23 region. The tumor stage of HCCs is indicated as I, II, III, and IV according to the TNM criteria. NI, no information. The pathology of HCCs and the etiological backgrounds of patients are indicated as follows: W, well differentiated; M, moderately differentiated; P, poorly differentiated; B, HBV-infected case; C, HCV-infected case; and N, non-HBV/non-HCV-infected. Symbols for LOH analysis are as follows: ○, retained; ●, LOH; and blank, no information. Methylation of *CDH1* promoter is indicated as hypermethylation (+) and nonmethylation (–). Expression of E-cadherin (*E-cad*) mRNA and protein is indicated as follows: +, lower than normal liver (<0.5); ++, same as normal liver (>0.5); +++, higher than normal liver (>2.0); and ND, not determined. The allele symbols for the SNP at –285 are 1 (C/C) and 1/2 (C/A).

Table 1 Characteristics of HCCs and E-cadherin alterations

	Tumor stage				Pathology ^a		
	I	II	III	IV	W	M	P
No. of cases	9	17	2	1	8	18	4
LOH (%)	3 (33)	8 (47)	2 (100)	0 (0)	1 (13)	11 (61)	1 (25)
Hypermethylation (%)	4 (44)	4 (24)	0 (0)	0 (0)	2 (25)	5 (28)	1 (25)

^a Pathology of HCCs was indicated as follows: W, well differentiated; M, moderately differentiated; and P, poorly differentiated.

type (well versus moderately differentiated type, $P = 0.02$ by the χ^2 test). The etiological backgrounds of the HCCs with LOH for this locus were as follows: (a) 2 of 5 (40%) were HBV infected; (b) 8 of 20 (40%) were HCV infected; and (c) 3 of 5 cases were non-HBV or non-HCV infected (60%).

Methylation of the 5'-CpG Island of *CDH1*. We next examined the methylation of the CpG island of the 5' region of *CDH1* by Southern blot analysis in 24 cases. The *Bam*HI/*Xba*I digest shows a 1184-bp fragment. By digestion with *Bam*HI/*Xba*I/*Hpa*II, a *Hpa*II-resistant band is detected when the CpG is methylated, whereas no obvious band is detected when the CpG is not methylated. Hypermethylation of 5'-CpG was observed in eight HCCs and in one nontumorous part of the liver that was affected with cirrhosis. Of these eight cases, three also showed LOH of *CDH1*. Therefore the CpG of the remaining allele was methylated in these cases. All eight cases showed a short *Hpa*II-

resistant band, which is thought to imply that methylation occurs at some of the CpG (Fig. 3). We could not detect a similarly sized *Hpa*II-resistant band by *Xba*I/*Bam*HI digestion, therefore not every CpG was methylated in these samples. Hypermethylation of the *CDH1* promoter was observed in the stage I and II tumors. The frequencies of hypermethylation were parallel among various histological types of HCCs (Table 1).

Expression of E-cadherin mRNA and Protein. The levels of E-cadherin mRNA and protein were analyzed in HCCs and adjacent nontumorous regions. We analyzed only a limited number of samples because some samples were exhausted, or the quality of RNA or cell extract was not suitable for analysis. In 23 cases, the expression of E-cadherin mRNA was determined by RNase protection assay (Fig. 4A). Down-regulation of E-cadherin mRNA was found in 10 of the 23 HCCs examined (see Fig. 1). Of these 10 cases, 8 showed LOH of the *CDH1*

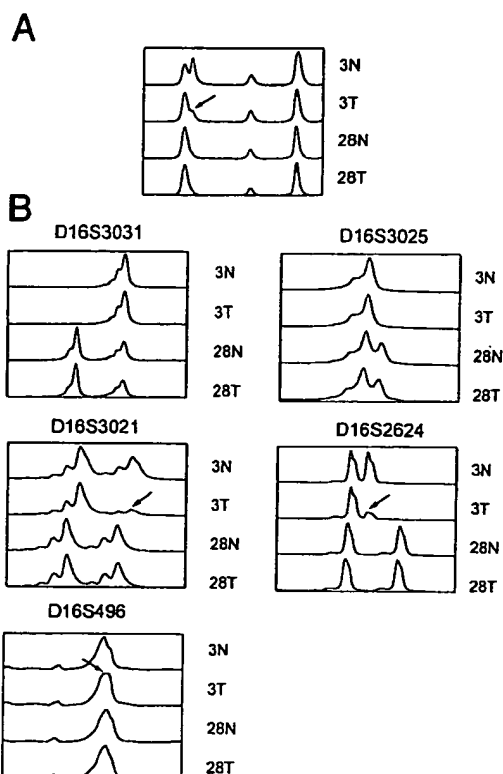


Fig. 2 LOH analysis of *CDH1* and adjacent 16q22-23 loci. LOH of (A) *CDH1* and (B) 16q22-23 was analyzed. A, SNP in *CDH1* +2076 was analyzed by fluorescence-based PCR-SSCP. B, microsatellite markers of 16q22-23 were analyzed. Peak heights were compared in HCC (T) and adjacent nontumorous liver (N). Case 3 shows LOH, but case 28 does not. The arrow indicates the lost allele.

region. Most nontumorous parts of the liver expressed the same level of E-cadherin mRNA as normal liver tissue. Therefore, down-regulation of E-cadherin mRNA is specific to HCCs. In 21 cases, the amount of E-cadherin was determined by immunoblot analysis (Fig. 4B). A reduced level of E-cadherin expression was observed in 10 cases. Of these 10 cases, 8 showed LOH of this locus, and 1 showed hypermethylation of CpG. The E-cadherin protein levels were comparable to the corresponding amount of mRNA. We detected reduced levels of E-cadherin mRNA and protein in only one case that showed hypermethylation of the promoter region without LOH. Two cases (cases 8 and 12) with LOH of *CDH1* but no change in either mRNA or protein levels probably arise because cells containing LOH included only a small tumorous population, based on estimation using the height of missing peaks (data not shown). The methylation of case 27, which showed a down-regulation of both E-cadherin mRNA and protein, was not examined.

Variation in the *CDH1* Promoter and Expression. We examined the allele patterns of the SNP at -285 in the *CDH1* promoter region because the -285A variant has been observed to have lower transcriptional activity than the -285C type (22). We found that seven patients had the -285A variant in one of the alleles (Fig. 1). The -285A variant showed no effect on *CDH1* expression in nontumorous liver tissues because various

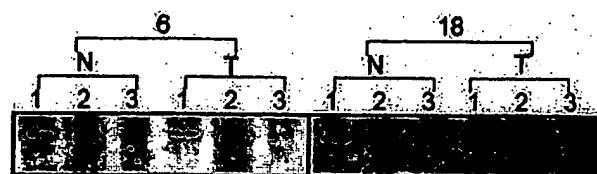


Fig. 3 Methylation of the *CDH1* promoter region. Methylation of 5'-CpG of *CDH1* was analyzed. DNA was digested with *Bam*HI/*Xba*I (Lane 1), *Bam*HI/*Xba*I/*Msp*I (Lane 2), and *Bam*HI/*Xba*I/*Hpa*II (Lane 3). Nontumorous livers (N) from both samples and HCC (T) of case 6 were not methylated, but HCC (T) of case 18 was partially methylated.

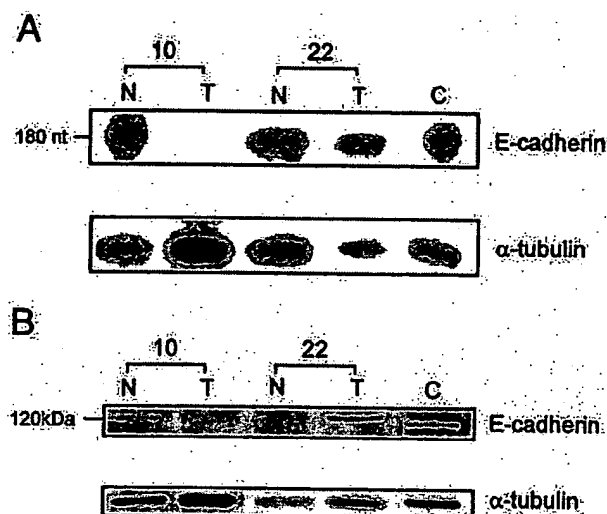


Fig. 4 Expression of E-cadherin mRNA and protein. A, expression of E-cadherin mRNA by RNase protection assay. A 180-nucleotide RNase-resistant band indicates E-cadherin mRNA. α -Tubulin mRNA was analyzed to assure the quality of mRNA. B, expression of E-cadherin by immunoblot analysis. A M_r 120,000 band shows E-cadherin. α -Tubulin was analyzed to assure the quality of cell extract. Reduced levels of both E-cadherin mRNA and protein in HCC are shown in case 10, but not in case 22. T, HCC; N, nontumorous liver.

levels of E-cadherin mRNA were observed in individuals having this allele. The pathology of the HCCs was grouped as 3 well-differentiated, 2 moderately differentiated, and 2 poorly differentiated types, and clinical stages were classified as 3 stage I cases, 3 stage II cases, and 1 stage III case. Thus, no significant correlation was found between allele patterns and tumor characteristics.

DISCUSSION

E-cadherin mediates cell-cell interaction in a calcium-dependent manner. The extracellular domain of E-cadherin is important for cell-cell interaction, and the intracellular domain binds to catenins that attach to the actin cytoskeleton. Loss of E-cadherin function disrupts cell-cell interaction and might induce the malignant progression of tumors. Recently, dysfunction of E-cadherin by genetic or epigenetic alterations has been reported in various kinds of epithelial cell tumors, such as breast, prostate, and stomach cancers (27-29).

In HCCs, there have been several studies of the down-regulation of E-cadherin by immunopathological methods (17, 18), but no comprehensive study of genetic or epigenetic changes of *CDH1* and their relation to its expression. The allelic deletion of *CDH1* and neighboring loci was found in 30–40% of Japanese HCCs in previous studies (30, 31) and in the present study. In Chinese HCCs, however, a high frequency (85%) of LOH of *CDH1* has been reported in HBV-infected HCCs (19), and the 16q region was also frequently deleted (70%) in HCCs in association with α -fetoprotein elevation (32). Here, however, LOH of *CDH1* occurred at similar frequencies in HBV-infected, HCV-infected, and non-HBV- or non-HCV-infected cases of HCC. Thus, LOH of 16q22–23 including *CDH1* was found less often in Japanese cases than in Chinese cases, and the frequency of LOH did not vary with the etiological background. The development of HCCs is therefore different in Japanese cases and in Chinese cases that are induced by exposure to carcinogenic toxins and HBV, although the deletion of *CDH1* is a critical factor in the progression of HCCs with different backgrounds.

According to immunohistochemical staining of tissues, there is a reduction in E-cadherin in tumors, with hypermethylation of the 5'-CpG island of *CDH1* in the breast, prostate, and liver (16–18). Thus, in many tumors, hypermethylation of the 5'-CpG island is a major cause of the down-regulation of *CDH1*. However, we found an obvious reduction of E-cadherin mRNA in only one of five HCCs with hypermethylation, which is a common pattern in HCCs with allelic deletion of the gene. Other reports indicate that the immunohistochemical staining pattern is heterogeneous in tumor cells of breast carcinomas with reduced E-cadherin, whereas strong staining is observed in all cells in normal or tumor tissues without reduced E-cadherin (33, 34). A recent study found that the methylation status of *CDH1* promoter and its protein expression vary among individual cells in the tumor (35). Down-regulation of E-cadherin mRNA and protein might therefore be heterogeneous in the tumor. Our study showed that the reduction of *CDH1* expression is not clear in HCCs with hypermethylation, suggesting that its expression is heterogeneous in these tumors. In contrast, LOH of *CDH1* was present in almost the entire cell population of tumors. Allelic deletion might therefore induce down-regulation of E-cadherin function more effectively than hypermethylation in a tumor.

The LOH of *CDH1* was found at a higher frequency in more malignant HCCs. All of the stage III HCCs, which had blood vessel invasion but no metastasis, carried allelic loss of *CDH1*. The LOH of *CDH1* in nontumorous livers was determined by comparing a peak height of SNP showing a heterozygous pattern with that of the normal heterozygote. Allelic deletion was found in 0 of 23 nontumorous livers showing a heterozygous pattern in any of the SNPs (data not shown). On the other hand, hypermethylation of the *CDH1* promoter was found frequently in less malignant HCCs, such as stage I HCCs. We also found methylation of *CDH1* promoter in one nontumorous cirrhotic liver, which was a precancerous hepatocyte. The down-regulation of E-cadherin by hypermethylation in the nontumorous liver might take place early in the development of HCCs. On the other hand, a stage IV HCC that had metastasized to lymph node, brain, and bone did not show LOH or hyper-

methylation in our study. Also, a recent study has shown that expression of E-cadherin is necessary for the intrahepatic metastasis of HCCs (36). Thus, the down-regulation of E-cadherin by hypermethylation might be involved in the early development of HCCs, whereas allelic deletion might contribute to the malignant progression of HCCs. On the contrary, the presence of E-cadherin appears to be necessary for completion of metastasis. The level of E-cadherin expression is therefore important to determine the characteristics of tumors.

The adenine variant of the -285 site in the promoter region correlates with reduced the transcriptional activity of *CDH1* (22). We are interested in whether such genetic variations determine the characteristics of tumors. No significant difference was found between the allele patterns and clinical stage of HCCs or the pathological findings. Also, we did not detect any significant correlation between the allele patterns and the expression of E-cadherin in nontumorous liver. E-cadherin mRNA or protein should be maintained at a stable level in the cells. In the present study, we found a novel SNP at -472 in the 5'-promoter region; a study of the contribution of this variation to the transcriptional activity of *CDH1* is under way.

We observed dysfunction of *CDH1*, although other genes localized on the 16q22–23 locus might be involved in hepatocellular carcinogenesis. For example, other cadherin family genes are localized in this region and could also be involved in the tumorigenesis of liver cells. The effect of mutation of β -catenin on the function of E-cadherin is also interesting because mutation of the Ser/Thr phosphorylation site of β -catenin is common in HCCs; moreover, changes in the tyrosine phosphorylation of β -catenin might induce changes in E-cadherin/catenin association (37, 38). Even if β -catenin protein was accumulated by mutation of the Ser/Thr residues or their neighboring site, the amount of E-cadherin in HCCs was not affected.⁵

In conclusion, down-regulation of E-cadherin by LOH or hypermethylation is critical to hepatocellular carcinogenesis. The level of E-cadherin expression may contribute to the determination of the characteristics of HCCs.

REFERENCES

1. El-Serag, H. B., and Mason, A. C. Rising incidence of hepatocellular carcinoma in the United States. *N. Engl. J. Med.*, 340: 745–750, 1999.
2. Bressan, B., Kew, M., Wands, J., and Ozturk, M. Selective G to T mutations of *p53* gene in hepatocellular carcinoma from southern Africa. *Nature (Lond.)*, 350: 429–431, 1991.
3. Hsu, I. C., Metcalf, R. A., Sun, T., Welsh, J. A., Wang, N. J., and Harris, C. C. Mutational hotspot in the *p53* gene in human hepatocellular carcinomas. *Nature (Lond.)*, 350: 427–428, 1991.
4. Hussain, S. P., and Harris, C. C. *p53* mutation spectrum and load: the generation of hypotheses linking the exposure of endogenous or exogenous carcinogens to human cancer. *Mutat. Res.*, 428: 23–32, 1999.
5. Murakami, Y., Hayashi, K., Hirohashi, S., and Sekiya, T. Aberrations of the tumor suppressor *p53* and retinoblastoma genes in human hepatocellular carcinomas. *Cancer Res.*, 51: 5520–5525, 1991.
6. Oda, T., Tsuda, H., Scarpa, A., Sakamoto, M., and Hirohashi, S. Mutation pattern of the *p53* gene as a diagnostic marker for multiple hepatocellular carcinoma. *Cancer Res.*, 52: 3674–3678, 1992.

⁵ Unpublished data.

7. Makino, R., Kaneko, K., Kurahashi, T., Matsumura, T., and Mitamura, K. Detection of mutation of the *p53* gene with high sensitivity by fluorescence-based PCR-SSCP analysis using low-pH buffer and an automated DNA sequencer in a large number of DNA samples. *Mutat. Res.*, 452: 83-90, 2000.
8. de La Coste, A., Romagnolo, B., Billuart, P., Renard, C. A., Buendia, M. A., Soubrane, O., Fabre, M., Chelly, J., Beldjord, C., Kahn, A., and Perret, C. Somatic mutations of the β -catenin gene are frequent in mouse and human hepatocellular carcinomas. *Proc. Natl. Acad. Sci. USA*, 95: 8847-8851, 1998.
9. Miyoshi, Y., Iwao, K., Nagasawa, Y., Aihara, T., Sasaki, Y., Imaoka, S., Murata, M., Shimano, T., and Nakamura, Y. Activation of the β -catenin gene in primary hepatocellular carcinomas by somatic alterations involving exon 3. *Cancer Res.*, 58: 2524-2527, 1998.
10. Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science (Washington DC)*, 251: 1451-1455, 1991.
11. Mareel, M., Boterberg, T., Noe, V., Van Hoorde, L., Vermeulen, S., Bruyneel, E., and Bracke, M. E-cadherin/catenin/cytoskeleton complex: a regulator of cancer invasion. *J. Cell. Physiol.*, 173: 271-274, 1997.
12. Guilford, P., Hopkins, J., Harraway, J., McLeod, M., McLeod, N., Harawira, P., Taite, H., Scoular, R., Miller, A., and Reeve, A. E. E-cadherin germline mutations in familial gastric cancer. *Nature (Lond.)*, 392: 402-405, 1998.
13. Oda, T., Kanai, Y., Oyama, T., Yoshiura, K., Shimoyama, Y., Birchmeier, W., Sugimura, T., and Hirohashi, S. E-cadherin gene mutations in human gastric carcinoma cell lines. *Proc. Natl. Acad. Sci. USA*, 91: 1858-1862, 1994.
14. Becker, K. F., Atkinson, M. J., Reich, U., Becker, I., Nekarda, H., Siewert, J. R., and Hofler, H. E-cadherin gene mutations provide clues to diffuse type gastric carcinomas. *Cancer Res.*, 54: 3845-3852, 1994.
15. Berx, G., Cleton-Jansen, A. M., Nollet, F., de Leeuw, W. J., van de Vijver, M., Cornelisse, C., and van Roy, F. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J.*, 14: 6107-6115, 1995.
16. Graff, J. R., Herman, J. G., Lapidus, R. G., Chopra, H., Xu, R., Jarrard, D. F., Isaacs, W. B., Pitha, P. M., Davidson, N. E., and Baylin, S. B. E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res.*, 55: 5195-5199, 1995.
17. Yoshiura, K., Kanai, Y., Ochiai, A., Shimoyama, Y., Sugimura, T., and Hirohashi, S. Silencing of the E-cadherin invasion-suppressor gene by CpG methylation in human carcinomas. *Proc. Natl. Acad. Sci. USA*, 92: 7416-7419, 1995.
18. Kanai, Y., Ushijima, S., Hui, A. M., Ochiai, A., Tsuda, H., Sakamoto, M., and Hirohashi, S. The E-cadherin gene is silenced by CpG methylation in human hepatocellular carcinomas. *Int. J. Cancer*, 71: 355-359, 1997.
19. Slagle, B. L., Zhou, Y. Z., Birchmeier, W., and Scorsone, K. A. Deletion of the E-cadherin gene in hepatitis B virus-positive Chinese hepatocellular carcinomas. *Hepatology*, 18: 757-762, 1993.
20. Hermanek, P., and Sorbin, L. H. TNM Classification of Malignant Tumors (Ed. 4), UICC. New York: Springer Verlag, 1987.
21. Berx, G., Becker, K. F., Hofler, H., and van Roy, F. Mutations of the human E-cadherin (CDH1) gene. *Hum. Mutat.*, 12: 226-237, 1998.
22. Li, L. C., Chui, R. M., Sasaki, M., Nakajima, K., Perinchery, G., Au, H. C., Nojima, D., Carroll, P., and Dahiya, R. A single nucleotide polymorphism in the E-cadherin gene promoter alters transcriptional activities. *Cancer Res.*, 60: 873-876, 2000.
23. Gyapay, G., Morissette, J., Vignal, A., Dib, C., Fizames, C., Millasseau, P., Marc, S., Bernardi, G., Lathrop, M., and Weissenbach, J. The 1993-94 Genethon human genetic linkage map. *Nat. Genet.*, 7: 246-339, 1994.
24. Dib, C., Faure, S., Fizames, C., Samson, D., Drouot, N., Vignal, A., Millasseau, P., Marc, S., Hazan, J., Seboun, E., Lathrop, M., Gyapay, G., Morissette, J., and Weissenbach, J. A comprehensive genetic map of the human genome based on 5,264 microsatellites. *Nature (Lond.)*, 380: 152-154, 1996.
25. Bussemakers, M. J., Giroldi, L. A., van Bokhoven, A., and Schalken, J. A. Transcriptional regulation of the human E-cadherin gene in human prostate cancer cell lines: characterization of the human E-cadherin gene promoter. *Biochem. Biophys. Res. Commun.*, 203: 1284-1290, 1994.
26. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.*, 162: 156-159, 1987.
27. Carter, B. S., Ewing, C. M., Ward, W. S., Treiger, B. F., Aalders, T. W., Schalken, J. A., Epstein, J. I., and Isaacs, W. B. Allelic loss of chromosomes 16q and 10q in human prostate cancer. *Proc. Natl. Acad. Sci. USA*, 87: 8751-8755, 1990.
28. Cleton-Jansen, A. M., Moerland, E. W., Kuipers-Dijkshoorn, N. J., Callen, D. F., Sutherland, G. R., Hansen, B., Devilee, P., and Cornelisse, C. J. At least two different regions are involved in allelic imbalance on chromosome arm 16q in breast cancer. *Genes Chromosomes Cancer*, 9: 101-107, 1994.
29. Umbas, R., Schalken, J. A., Aalders, T. W., Carter, B. S., Karthaus, H. F., Schaafsma, H. E., Debruyne, F. M., and Isaacs, W. B. Expression of the cellular adhesion molecule E-cadherin is reduced or absent in high-grade prostate cancer. *Cancer Res.*, 52: 5104-5109, 1992.
30. Tsuda, H., Zhang, W. D., Shimamoto, Y., Yokota, J., Terada, M., Sugimura, T., Miyamura, T., and Hirohashi, S. Allele loss on chromosome 16 associated with progression of human hepatocellular carcinoma. *Proc. Natl. Acad. Sci. USA*, 87: 6791-6794, 1990.
31. Fujimori, M., Tokino, T., Hino, O., Kitagawa, T., Imamura, T., Okamoto, E., Mitsunobu, M., Ishikawa, T., Nakagama, H., Harada, H., et al. Allelotype study of primary hepatocellular carcinoma. *Cancer Res.*, 51: 89-93, 1991.
32. Yeh, S. H., Chen, P. J., Lai, M. Y., and Chen, D. S. Allelic loss on chromosomes 4q and 16q in hepatocellular carcinoma: association with elevated α -fetoprotein production. *Gastroenterology*, 110: 184-192, 1996.
33. Moll, R., Mitze, M., Frixen, U. H., and Birchmeier, W. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *Am. J. Pathol.*, 143: 1731-1742, 1993.
34. Siitonen, S. M., Kononen, J. T., Helin, H. J., Rantala, I. S., Holli, K. A., and Isola, J. J. Reduced E-cadherin expression is associated with invasiveness and unfavorable prognosis in breast cancer. *Am. J. Clin. Pathol.*, 105: 394-402, 1996.
35. Graff, J. R., Gabrielson, E., Fujii, H., Baylin, S. B., and Herman, J. G. Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J. Biol. Chem.*, 275: 2727-2732, 2000.
36. Osada, T., Sakamoto, M., Ino, Y., Iwamatsu, A., Matsuno, Y., Muto, T., and Hirohashi, S. E-cadherin is involved in the intrahepatic metastasis of hepatocellular carcinoma. *Hepatology*, 24: 1460-1467, 1996.
37. Matsuyoshi, N., Hamaguchi, M., Taniguchi, S., Nagafuchi, A., Tsukita, S., and Takeichi, M. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.*, 118: 703-714, 1992.
38. Roura, S., Miravet, S., Piedra, J., Garcia de Herreros, A., and Dunach, M. Regulation of E-cadherin/catenin association by tyrosine phosphorylation. *J. Biol. Chem.*, 274: 36734-36740, 1999.